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EFFECTS OF AMINO ACIDS, MAGNESIUM, AND MOLLUSCAN EXTRAPALLIAL FLUID ON CRYSTALLIZATION OF CALCIUM CARBONATE: *IN VITRO* EXPERIMENTS

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Abstract

The extrapallial fluid (EPF), the fluid component of the shell-forming system of molluscs, has been examined for its effect on the rate of $CaCO_3$ crystal formation *in vitro*. The medium was an artificial inorganic extrapallial fluid supersaturated with respect to $CaCO_3$. EPF of the bivalves *Crassostrea virginica* and *Mercenaria mercenaria* strongly inhibited $CaCO_3$ crystallization in high dilution. The inhibitory material had negatively charged groups as indicated by the removal of inhibition subsequent to passage through a DEAE-Sephadex column and the restoration of inhibition in material eluted from the column with 2.0 *M* NaCl. Polyaspartic and polyglutamic acids with COO^- groups also inhibited crystal formation whereas polyamino acids lacking those groups and free amino acids were without effect. The inhibition by the acidic polyamino acids suggests that polypeptides known to be present in EPF are one possible cause of inhibition of crystal formation by EPF. Mg present in EPF was also strongly inhibitory. The observed inhibition of *in vitro* crystallization by EPF and Mg indicates that these substances could be factors in governing $CaCO_3$ deposition rate in molluscan shell.

Introduction

A wide range of studies has been devoted to the effects of inorganic ions and organic compounds on the rates of formation of crystals of calcium carbonate and calcium phosphate. These varied studies have attempted to gain insight on the mechanisms controlling crystal formation in natural waters, in invertebrates and vertebrates, and in marine algae.

In molluscs and marine algae, which have calcium carbonate skeletons, the organic compounds of the skeletons have been given attention because of their possible roles in crystal nucleation (Crenshaw, 1972a; Weiner and Hood, 1975; Crenshaw and Ristedt, 1976; Weiner, 1979; Samata *et al.*, 1980; Weiner and Traub, 1981), inhibition of crystal growth (Crenshaw and Ristedt, 1976; Wilbur, 1976; Weiner, 1979; Weiner and Traub, 1981), and crystal orientation (Weiner and Traub, 1981). By means of an *in vitro* method, inhibition of $CaCO_3$ crystal formation has been demonstrated for the organic material in sea water (Chave and Suess, 1967, 1970), for the soluble organic matrix of the shell of the oyster *Crassostrea virginica* (Wheeler *et al.*, 1981); and for the polysaccharide associated with coccoliths of the alga *Emiliania huxleyi* (Borman *et al.*, 1982). However, nucleation by individual invertebrate proteins has not been demonstrated. In addition to investigations on calcium carbonate crystallization, a number of compounds and conditions have been studied for their effects on the rate of *in vitro* calcium phosphate crystallization (Boulet and Marier, 1961; Bachra and Fisher, 1969; Francis *et al.*, 1969; West and Storey, 1972; Robertson,

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AIEF, artificial inorganic extrapallial fluid; EFP, extrapallial fluid.

1973; Termine and Eanes, 1974; Termine and Conn, 1976; Moreno *et al.,* 1979; Boskey and Posner, 1980; Termine *et al.,* 1980).

In molluscan shell, the calcium carbonate crystals are formed on the inner shell surface in association with the extrapallial fluid and an organic matrix, both derived from secretion of the mantle epithelium. The extrapallial fluid contains inorganic ions, including calcium and magnesium, and various organic compounds, including proteins, carbohydrates, and amino acids (see Wilbur and Saleuddin, 1983). The initiation and inhibition of $CaCO_3$ crystallization in molluscs, have been attributed to the polypeptide and carbohydrate moieties of the organic matrix. Weiner and Traub (1981) hypothesized that Ca-binding COO^- groups of polypeptide chains may initiate or inhibit crystal growth, depending upon their spacing relative to the spacing of Ca of the $CaCO_3$ crystal lattice. Crenshaw and Ristedt (1976) have suggested that sulfated polysaccharide groups which bind calcium may initiate $CaCO_3$ crystal nucleation by the process of ionotropy. However, if the material bearing the sulfated groups is secreted by the mantle on growing crystals, the sulfated groups are thought to attach to calcium on the crystal surface lattice, thereby inhibiting further additions of ions and halting crystal growth.

In the present study, two sets of experiments are reported. The first set examines the influence of the extrapallial fluid in initiating or inhibiting $CaCO_3$ crystallization *in vitro.* Magnesium, a component of extrapallial fluid and an inhibitor of $CaCO_3$ crystallization in sea water (Pytkowicz, 1965) has also been studied for its effect on crystallization rate. The second set of experiments evaluates the effects of charges associated with organic molecules on $CaCO_3$ crystallization rates. Extrapallial fluid from which negatively charged substances were removed and free and polyamino acids differing in charge have been studied. A solution containing the major inorganic components of molluscan extrapallial fluid was used as a test system.

The experiments on free and polyamino acids were undertaken in collaboration with Dr. C. S. Sikes.

Materials and Methods

Media

Artificial inorganic extrapallial fluid (AIEF) was prepared based on analyses of the inorganic components of molluscan extrapallial fluid (Crenshaw, 1972b; Wada and Fujinuki, 1976). Ionic concentrations are assembled in Table I. All reagents used in AIEF were analytical grade with the exception of highly pure $CaCl_2$ obtained from Sigma Chemicals. Solutions were prepared weekly and kept at 4°C.

Crystallization assay

Thirty ml of AIEF were placed in a 100-ml round bottom flask. One ml of the test substance in glass distilled water or 1.0 ml glass distilled water (control) was added and the flask was stirred by means of a magnetic stirrer and a 3 × 1.5 mm teflon stirring bar. A pH electrode secured in the flask by a well-fitting rubber collar was immersed in the fluid. The pH of the solution was adjusted to 8.60 with NaOH to offset the pH decrease that occurs when the $CaCl_2$ is added. The flask was tightly stoppered to prevent exchange with outside air, and monitored by means of a chart recorder connected to an expanded scale pH meter (Fisher Accumet 107). The pH was recorded continuously beginning with the addition of 0.9 ml of 1.0 *M* $CaCl_2$ which supersaturated the solution and lowered the pH to 8.33. The final Ca concentration was 28.21 m*M*. During most experiments, the temperature was kept at

TABLE I

Inorganic components of normal and experimental extrapallial fluids

ion	analysis 1*	analysis 2**	our AIEF***
	m*M*	m*M*	m*M*
Na^+	441–444	422.8–431.5	442
K^+	9.4–9.6	9.6–12.7	9.5
Ca^{2+}	10.65–11.6	9.5–9.9	28.2
Mg^{2+}	57–60	48.2–50.7	47.2
Cl^-	472–480	520.1–552.6	476
SO_4^{2-}	46.1–47.3	26.2–33.3	47.2
HCO_3^-	4.2–5.2 (CO_2)	2.4–5.2	7.6
pH	7.33–7.41	8.4–8.53	7.5

* Analysis 1 from Crenshaw, 1972b. Range of data from 3 species of marine molluscs.
** Analysis 2 from Wada and Fujinuki, 1976. Range of data from 4 species of marine molluscs.
*** AIEF, artificial inorganic extrapallial fluid used in these experiments.

21 C ± 1°C. In a variation of this experiment, 1.0 ml of the test substance in a solution of AIEF was added to the flask when the pH reached 8.15 to assess the effect on crystals which were already forming.

Crystallization of $CaCO_3$ was followed by measurements of pH change resulting from protons released in the reaction

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+.$$

This method is similar to that used by Pytkowicz (1965), Chave and Suess (1970), Wheeler *et al.* (1981), Sikes and Wheeler (1983), and Borman *et al.* (1982). During nucleation and crystallization, the pH decreased from pH 8.33 to approximately pH 7.5. In so doing, the pH was within the range reported for extrapallial fluids under normal conditions (Table I). The length of time prior to initiation of crystal nucleation and pH decrease is termed induction time (Garside, 1982). For comparative purposes we have chosen to represent this length of time as a function of both the induction period and the crystallization rate. An illustration of our method is shown in Figure 1. Mean induction times were obtained for each of the test substances and their standard deviations were calculated.

The course of crystallization was also followed in a few experiments by simultaneous measurements of optical changes (Termine and Eanes, 1974) and pH. Similar, but not identical, changes occurred in both.

Extrapallial fluid experiments

Specimens of the oyster *Crassostrea virginica* and the clam *Mercenaria mercenaria* were collected near the Duke University Marine Laboratory at Beaufort, North Carolina. Extrapallial fluid (EPF) was withdrawn immediately by prying open the valves and inserting a needle under the mantle edge into the central extrapallial space and collecting the fluid in a sterile syringe. Samples thought to be contaminated by sea water were not used. EPF from several animals was pooled, kept on ice, and the first samples were used on the day of collection. Remaining samples were maintained on ice in a refrigerator at 4°C. The measured characteristics of these samples remained stable over 7 days.

Various amounts of EPF were added to AIEF and induction times of crystallization were measured. To ascertain whether the observed inhibitory activity of EPF was due in part to the presence of negatively charged groups, the fluid was passed through

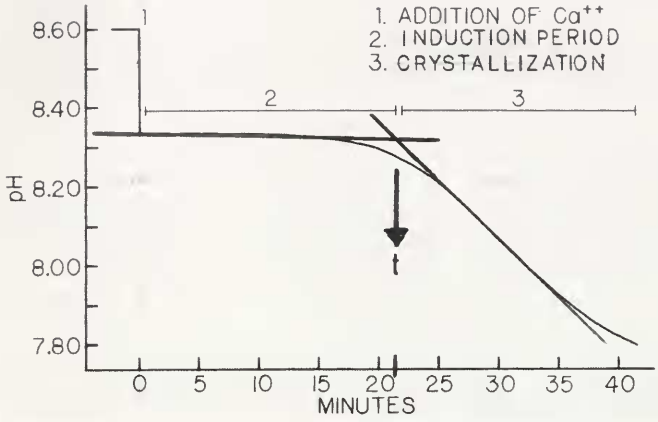

FIGURE 1. Chart recording of an actual control curve showing the 3 phases of *in vitro* $CaCO_3$ crystallization in artificial inorganic extrapallial fluid. Induction time (t) is indicated by the intersection of the 2 lines drawn along phases 2 and 3 on the graph.

a 9 × 20 mm DEAE-Sephadex column. Preliminary experiments were undertaken to determine the appropriate pH of the buffer and resin. A pH of 7.0 was selected and 2 columns were prepared with resin allowed to swell in AIEF, pH 7.0, for 2 days. The columns were then poured and washed with 50 volumes of AIEF and rewashed with 20 volumes 0.05 Tris buffer at the same pH. Measurement of the induction time of EPF, (200 μl EPF per ml of 0.05 *M* Tris buffer), was carried out. A 1.35 ml sample of this dilution was then applied to each of 2 columns and a 1.25 ml aliquot was collected from each. This material was assayed against a control column-run sample which contained buffer alone. The columns were then eluted with 1.35 ml of 0.5 *M* NaCl in Tris buffer, and a second elution was made using 2.0 *M* NaCl in 0.05 *M* Tris buffer. Samples of unbound and eluted materials were assayed in duplicate.

Magnesium Experiments

The effect of Mg on the induction time of crystallization was measured in magnesium-free AIEF to which $MgCl_2$ was added to give final concentrations of 0.5–120 m*M*.

Amino acid experiments

Seven free and 5 polyamino acids (Sigma Chemicals) were studied for their effects on $CaCO_3$ crystallization. The free amino acids included aspartic and glutamic acids, glycine, leucine, lysine, histidine, and taurine. Polyamino acids tested were polyaspartic and polyglutamic acids, polyglycine, polyleucine, polylysine, and polyhistidine. All free amino acids were tested at concentrations up to 1.47×10^{-4} *M*.

Because the polyamino acids differed greatly in their degree of polymerization, concentrations were standardized by preparing dilutions to contain equal numbers of amino acid residues. Concentrations are expressed in this way to allow ready comparison between the different polyamino acids regardless of their degree of polymerization. The polyamino acids were tested at as nearly equivalent concentrations as possible except in the case of polyaspartic and polyglutamic acids which inhibited crystallization so markedly that direct comparison with other polyamino acids was impractical. In these cases, lower concentrations were investigated to determine the lowest concentrations capable of producing significant inhibition of $CaCO_3$ crystallization.

Results

All experimental and control runs produced sigmoid curves between the point marked by the addition of $CaCl_2$ (pH 8.33), and pH 7.80, the point at which $CaCO_3$ formation was essentially complete (Fig. 1). Artificial inorganic extrapallial fluid (AIEF) to which no $CaCl_2$ had been added gave a straight line trace on the chart recorder for a week or more with a pH decrease of only a few hundredths of a pH unit.

The induction times of crystallization were compared in samples of extrapallial fluid prior to and following passage through DEAE-Sephadex columns which bind compounds with negatively charged groups. Whole EPF was assayed at concentrations ranging from 0.1 μg/ml (7 μl) to 14.0 μg/ml (980 μl). At 3 μg/ml (210 μl EPF/ml) induction time increased to ≃800% of control time. At 14.0 μg/ml crystallization had not taken place after 2 days. The following experiment illustrates the results after the passage of EPF through DEAE columns. A sample containing 200 μl (2.9 μg/ml) of EPF in 0.05 *M* Tris buffer, pH 7.0, had an induction time of >3 h. The induction time was reduced to 18.5 min after this material was passed through a DEAE column. This material contained a Mg concentration roughly equivalent to the concentration found in the control material as the column does not effect the cationic components of material applied. Since the mean control time for the sample without EPF was 20.5 min, it appears that all inhibitory activity had been removed by the column. After elution of the column with 2.0 *M* NaCl, the induction time of the eluate was again >3 h, as compared to a 15 min control elution, demonstrating recovery of the bound inhibitory material from the column.

Induction time of crystallization increased with Mg concentrations of 5 m*M* Mg and above (Table II). At 47 m*M*, the equivalent of total Mg present in the extrapallial fluid of marine bivalves (Crenshaw, 1972b; Wada and Fujinuki, 1976), the induction time was several fold greater than that produced in the absence of Mg.

All free amino acids resulted in mean induction times which were not significantly different statistically from the control mean even at the highest concentrations tested (1.47×10^{-4} m*M*). Polyamino acid solutions containing the same number of residues per volume were compared. Polyaspartic and polyglutamic acids produced significantly greater induction times at concentrations equal to or lower than those of the corresponding free amino acids. Polyaspartic acid was inhibitory at concentrations of 7.36×10^{-8} *M* residues and above. The average length of induction time as a function of residue concentration for polyaspartic and polyglutamic acids is shown in Table III. These data imply an exponential increase in induction time with increasing polyamino acid concentration. Polyhistidine, polyglycine, and polylysine lacking carboxyl groups produced no significant increase in induction times even when tested at concentrations 100 times greater than those which produced a minimal effect with

TABLE II

Summary of data from in vitro $CaCO_3$ crystallization experiments

Test material	Concentration	Induction time in minutes (+S.D.)
control	0	21.5 (±7.4)
magnesium	0.5 m*M*	26.0 (±4.0)
	5.0 m*M*	48.5 (±1.8)
	29.2 m*M*	55.7 (±4.3)
	47.2 m*M*	190.0 (±2.0)
	117.0 m*M*	>23 h
whole EPF*	Tris buffer p̄ column control	20.5 (±2.0)
	200 μl/ml. buffer	>3 h
	EPF post column	18.5 (±3.5)
	EPF p̄ column elution (2.0 *M* NaCl)	>3 h
	Control elution (2.0 *M* NaCl)	15.0

* Extrapallial fluid obtained from *Mercenaria mercenaria.*

the negatively charged polyglutamic and polyaspartic acids. Our results with free amino and polyamino acids support those of Sikes and Wheeler (1983), carried out in artificial sea water adjusted to pH 9.5. These investigators observed that only negatively charged polyamino acids produced an inhibitory effect on mineralization.

In a variation of the experiments reported above, polyglutamic acid (pH adjusted to 8.15) when added to the flask at a concentration of 1.47×10^{-6} *M* residues *after* crystallization had begun, immediately arrested crystal formation and produced an induction time which was significantly longer than the control (Fig. 2).

DISCUSSION

Artificial inorganic extrapallial fluid (AIEF) to which sufficient Ca has been added, will form crystals of $CaCO_3$ after an induction period which depends upon conditions in the fluid (see Mullin, 1972). Using inorganic extrapallial fluid (AIEF) with added Ca, we have demonstrated that extrapallial fluid (EPF) in high dilution markedly decreases the rate of $CaCO_3$ crystallization. This inhibition was removed by passage

TABLE III

Mean induction times for various concentrations of polyaspartic and polyglutamic acids

Sample	Concentration*	Mean induction time in minutes (+S.D.)
Control (no amino acids)	0	23.5 (±8.3)
Polyaspartic acid	7.36×10^{-9} *M*	27.0 (±0.28)
	1.47×10^{-8} *M*	24.7 (±1.4)
	**7.36×10^{-8} *M*	52.3 (±4.0)
	1.47×10^{-7} *M*	115.7 (±25.9)
	7.36×10^{-7} *M*	48.0 h (±2.2h)
Polyglutamic acid	7.36×10^{-8} *M*	25.2 (± 4.5)
	**1.47×10^{-7} *M*	124.0 (±5.0)
	1.47×10^{-4} *M*	18.1 h

* Concentrations expressed in *M* residues.

** Concentration at which inhibition can be detected

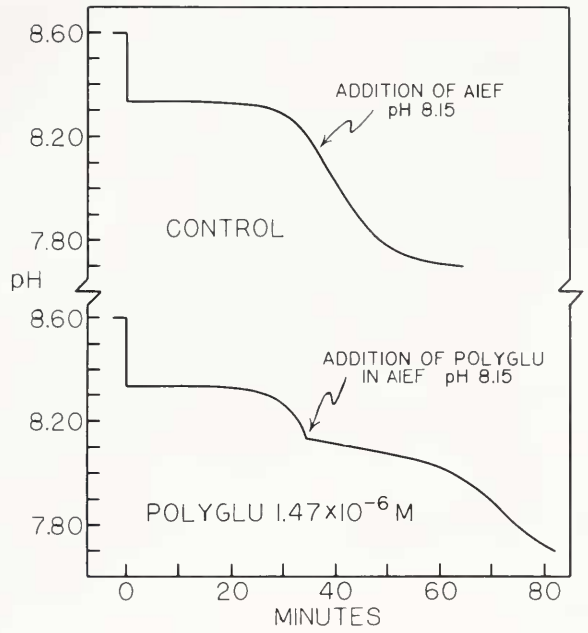

FIGURE 2. Time course of $CaCO_3$ crystallization in artificial inorganic extrapallial fluid (AIEF). Lower curve shows temporary retardation of crystal growth on addition of polyglutamic acid in AIEF, pH 8.15, after crystallization had begun. Upper curve demonstrates the absence of inhibition upon addition of AIEF, pH 8.15, without polyglutamic acid at a corresponding time.

through DEAE-Sephadex indicating that the inhibition was produced by negative groups. Carboxyl groups of proteins and carbohydrates could be involved since proteins (Pietrzak *et al.,* 1973) and carbohydrate (Misogianes and Chasteen, 1979) are present in extrapallial fluid. However, other anionic groups of polypeptides including phosphate (see Sikes and Wheeler, 1983) and anionic groups of polysaccharides and organic acids of metabolism cannot be excluded. Both COO^- and SO_4^{2-} have been considered possible inhibitors of crystal growth (Crenshaw and Ristedt, 1976; Weiner and Traub, 1981). Borman *et al.* (1982) found that the carboxyl groups, but not the ester sulfate groups, of the polysaccharide of coccoliths of *Emiliana* were important in inhibiting $CaCO_3$ crystallization *in vitro.*

Wheeler *et al.* (1981) and Sikes and Wheeler (1983) found that soluble shell matrix inhibited the formation of $CaCO_3$ crystals. This was presumed to be due to the negatively charged groups associated with protein in *Crassostrea.* Inhibition of crystal formation *in vitro* by polyaspartic and polyglutamic acids which have multiple COO^- groups (Sikes and Wheeler, 1983; this study) supports this explanation. The inhibition of crystallization by polyamino acids with acidic residues, but not by equivalent concentrations of the corresponding free amino acids, demonstrates that multiple COO^- groups in a polypeptide chain are required for inhibition (Sikes and Wheeler, 1983; Wilbur and Bernhardt 1982). Attachment of the chains to crystal nuclei and crystal surfaces probably interferes with growth of the lattice. Complete inhibition of crystal formation with sufficient concentrations of extrapallial fluid and acidic polyamino acids observed in the present experiments is presumably the result of the binding of acidic groups to crystal nuclei (Borman *et al.,* 1982). Calculations indicated that the maximum calcium reduction in our system through binding by free and

polyamino acids did not reduce the free calcium content of the solution below supersaturation and did not exceed 0.4% change in calcium concentration.

Magnesium was found to decrease the rate of crystallization. In extrapallial fluid of marine bivalves the total Mg concentration is 49–60 m*M* (Crenshaw, 1972b; Wada and Fujinuki, 1976). The *in vitro* induction time for $CaCO_3$ in this range of concentrations was prolonged (Table II), and it follows that the Mg concentration in EPF may also retard crystal formation *in vivo*. Pytkowicz (1965) found that Mg also extended the induction time of the precipitation of $CaCO_3$ in sea water. In both sea water and EPF of marine bivalves, the concentration of Mg is some 5-fold that of Ca (Crenshaw, 1972b; Wada and Fujinuki, 1976). Pytkowicz (1965) points out that under such conditions collisions producing Mg-Ca-CO_3 aggregates will be more frequent than collisions resulting in $CaCO_3$. Accordingly, induction time will be increased.

The observed inhibitory effects of extrapallial fluid, negatively charged polyamino acids, and Mg on $CaCO_3$ crystallization *in vitro* raises the question as to the relationship between the experimental findings and $CaCO_3$ deposition by the organism. It will be evident that an extrapolation from the *in vitro* results to the mollusc is scarcely permissible in view of the differences between the two systems and the probable changes in the extrapallial fluid during the course of shell deposition. These differences notwithstanding, it may be that the inhibitory effects of organic compounds with negative groups and Mg could play a role in governing the rate of $CaCO_3$ deposition in molluscan shell.

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